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ENHANCED AVAILABILITY OF BIOFUEL AND BIOMASS COMPONENTS IN ASPERGILLUS NIGER AND ASPERGILLUS FUMIGATUS TREATED RICE HUSK

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Abstract

Aspergillus fumigatus (AF) and *Aspergillus niger* (AN) were isolated from consortia of other microorganisms from an over 8 months decomposing rice husk dump using potato dextrose agar (PDA). Pure strains of individual *Aspergillus* species were obtained and identified. Measured quantities of freshly processed rice husk in Mandle's medium were heat pre-treated in an autoclave at 121^{o}C for 20 minutes, cooled and inoculated with the fungi. Seven days fungal treated rice husks were assayed for the following biomass content: Carbohydrate (total sugar), simple sugar, non-reducing sugar, cellulose, lignin and protein. *Saccharomyces cerevisae* from baker's yeast and from palm wine were introduced into some of the fungal treated rice husks and fermented for 7 days for their bio-ethanol content. All samples data were in triplicate before analysis. The data were statistically analyzed using analysis of variance (ANOVA- SPSS 16) data package). Differences were considered significant at $p < 0.05$. Data were presented in percentages.

The result obtained in the work showed that rice husks treated with *Aspergillus fumigatus* had the highest cellulose (45 ± 3.31 %), hemicelluloses (31 ± 3.00 %), carbohydrate (19.52 ± 10.05 %), reducing sugar(2.60 ± 0.30 %) and non reducing sugar (16.92 ± 9.75 %) yields; ethanol from palm wine yeast yield was 6.60 ± 0.48 % while bakers yeast yielded 5.60 ± 0.42 % ethanol. Diculture combination of *Aspergillus fumigatus* and *Aspergillus niger* (AF+AN) gave the greatest total lignin value of 33.34 ± 3.14 %. The values obtained in the various bio-fuel and biomass components of fungal monocultures of *Aspergillus fumigatus* (AF) and *Aspergillus niger* (AN) treated rice husk had no significant difference in increase of product yield at $P > 0.05$. However, there were significant differences in product yield between the fungal treated rice husks and those of the controls at $P < 0.05$. The optimized condition involved in bio-product estimation showed that 30^oC and pH 5 gave the maximum carboxymethylcellulase activity in crude enzyme of products respectively. It was discovered that heat pre-treated rice husks with additional hydrolysis using the fungi (*Aspergillus fumigatus* and *Aspergillus niger*) showed increase in biomass and biofuel yields.

Keywords: *Aspergillus niger*, *Aspergillus fumigatus*, Biomass, Biofuel, Rice husk

Introduction

Most of the current agro-waste sectors research is channeled towards the effective utilization of byproduct from processed agricultural crops. The need to utilize bioproducts from agricultural wastes stem from the fact that the growing population of the world could be better served when those by-products otherwise considered as wastes are reintegrated for the generation of new product as well as enhancement of greener environments. The occupation of large land mass by agro-wastes has been a great environmental challenge of the 21st century. According to Grigorevski-Lima (2009) lignocellulosic residues from agriculture and forestry have potential as cheap and renewable feedstocks for large-scale production of fuels and chemicals. The agricultural wastes are composed essentially of cellulosic or lignocellulosic matter. These are considered to be the cheapest source for the production of different utilizable products throughout the world (Ali *et al*., 1991). Cellulose is commonly degraded by enzyme called cellulase. Complete enzymatic hydrolysis of cellulose requires synergistic action of 3 types of enzymes, namely cellobiohydrolases, endoglucanases or carboxymethylcellulase (CMCase) and B glucosidases (Bhat, 2000). All four classes of enzymes have been identified in *Aspergillus* (de Vries & Visser, 2001). Filamentous fungi particularly *Aspergillus* and *Trichoderma* spp., are

well known efficient producers of cellulases (Peij *et al.*, 1998). Lignocellulose is a complex substrate and its biodegradation is not dependent on environmental conditions alone, but also the degradative capacity of the microbial population (Waldrop *et al*., 2000; Malherbe and Cloet, 2002). Lignocellulose biodegradation is essentially a race between cellulose and lignin degradation (Reid, 1989; Malherbe and Cloet, 2002). Fungi with restricted metabolic capabilities develop mutualistic relationships in degrading cellulose, lignin etc. (Rayner and Boddy, 1988; Malherbe and Cloet, 2002). Most fungi are capable cellulose degraders and such fungi produce active polymer degrading enzymes, including cellulases and xylanases (Hodrova *et al*., 1998; Malherbe and Cloet, 2002). Their cellulases are among the most active reported to date and able to solubilise both amorphous and crystalline cellulose (Wubah *et al*., 1993; Malherbe and Cloet, 2002). Most microorganisms utilize lignin as carbon sources in their catabolic activities. The hydrolysis of cellulose can make available the sugar component which will eventually be converted to fine products such as ethanol and other organic acids through fermentation by yeast. This research is aimed at establishing the effect of heat treatment and fungal enzymatic reduction of rice husk to the various bio-components as well as the final bio-fuel product which is ethanol.

Materials and method

Plant sample (Rice Husk)

Samples of fresh processed rice husks and 8 months decomposing rice husk were collected from Adani Rice Integrated Resources Nig. Ltd., Adani in Uzo-Uwani Local Government Area of Enugu State, Nigeria. All samples were kept in air tight cellophane bags before use.

Isolation of fungi

Isolation of fungi from decomposing rice husk was carried out using Potato Dextrose Agar (PDA) as the inoculation medium. The method used was the dilution plate technique.

Screening, Isolation and characterization of fungi

Decomposing rice husk (1g) was added to 9 ml of sterile distilled water in a beaker and mixed thoroughly. This served as the stock for the isolation of the fungi. Serial dilution of the sample was carried out by pipetting 1 ml of the stock solution into another 9 ml of distilled water. The sample suspension was further diluted to 10^{-6} ml. From 10^{-6} serial diluted (fungal sources) stock, 0.1 ml was pipetted into five different petri dishes containing freshly prepared potato dextrose agar with inclusion of streptomycin/chloramphenol at 50^{o}C on an alcohol sterilized bench.

Spreading of inoculum was done by the pour plate method followed by gentle agitation to enable uniform spread. This was carried out using standard sterilization techniques in the presence of gentle Bunsen flame. The inoculated plates were incubated in a microbial laboratory incubator at room temperature of 38 ± 0.06 ^{o}C for 5 days. Growth was monitored daily and identification of the various fungal colonies carried out by microscopic cell observation, colony morphology and biochemical tests. Characteristics such as size, surface appearance, texture, sporing structure and colour of the colonies (picture of the various colonies were also taken and compared with standards for identification) were carried out. Subcultures (3 times for each identified colony) from the various plates were carried out by aseptically transferring each independently identified colony isolate into other potato dextrose agar slants (containing antibiotics) until pure fungal strains were obtained (for any batch the incubation was at room temperature recorded for 5 days) as seen in figure. 1. Pure fungal isolates were stored in culture tubes plugged with cotton wool in a refrigerator at 4^{o}C for further use. Subsequent culturing of the identified pure fungal strains were carried out using PDA agar in Petri dishes with the inclusion of streptomycin/chloramphenol after autoclave to be followed by incubation at room temperature for 6 days before use.

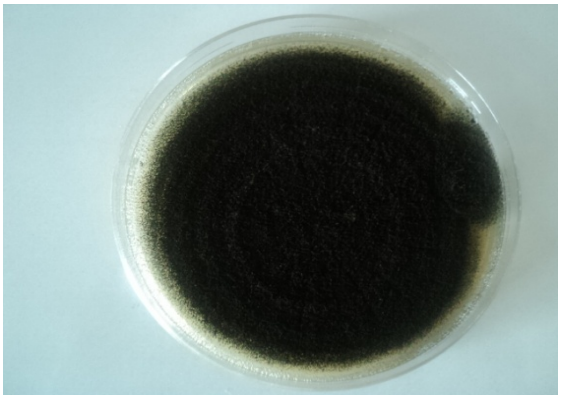

Figure 1: Pure culture of *Aspergillus spp*

Preparation of culturing and fermentation medium

For the cultivation of the fungi and hydrolysis of the rice husk, Mendle's medium was prepared as reported by Patel *et al*, (2007). One litre of Mandle's medium composes the following mineral salt ingredients: $(NH_4)_2SO_4$ (1.4g), KH_2PO_4 (2.0g), Urea (0.3g), $CaCl_2.2H_2O$ (0.4g), $MgSO_4.7H_20$ (0.6g), $MnSO_4.H_2O$ (1.0mg)**,** $ZnSO_47H_2O$ (1.4mg), $FeSO_4.7H_2O$ (5.0mg)**,** $CoCl_2.6H_2O$ (3.7mg), Protease-peptone (0.75g)**,** Tween80 (2.0mg). The medium (rice husk in Mandle's medium) was sterilized at 121^{o}C for 20 min. and the pH adjusted to 5.5.

Experimental design for the fungal treatment of rice husk

The experimental method was modification of Patel *et al*, (2007). Into each 500ml conical flask used in the experiment, 20g of rice husks were weighed (total of 5 samples) and 400ml of Mandle's medium introduced. Sterilization of the various conical flasks plugged with cotton and covered with aluminium foil was carried out using an autoclave at 121^{o}C for 20 minutes and cooled. Each conical flask except the controls (C1= non fungal but heat treated sample; C2 = non fungal, non heat treated sample) was inoculated with the fungi by addition of 10 ml of 0.1 % Tween 80 into PDA Petri dishes of pure fungal isolates both as monoculture: *Aspergillus fumigates* (AF), *Aspergillus niger* (AN) and di-culture (inoculates): combination of *Aspergillus fumigates* and *Aspergillus niger* (AF+AN) after proper labeling and aseptically transferring their conidia and spores into sterile tubes with the aid of sterilized cotton swabs. From each sterile tube, 1 ml fungal suspension was used for the inoculation. The flasks were incubated at room temperature for 7 days with 90 minutes daily agitation. The mycelia were separated by filtration through Whatman filter paper No. 1 and discarded. The filtrate was recovered while the treated rice husk residues were dried on filter paper using an oven temperature of 105^{o}C for 10 minutes. From each treatment, 1g was used to determine carbohydrate, reducing sugar and non-reducing sugar, cellulose, total sugar (carbohydrate), hemicelluloses, lignin and protein content in triplicates. The remaining rice husks (treatment and control samples) were inoculated with baker's yeast and yeast from palm wine and left to ferment for 7 days. Thereafter, ethanol yield was estimated.

Analysis with filtrate

Cellulase Enzyme Assay

The carboxymethylcellulase (CMC) activities of day 7 supernatants of cultures from the Mandel's fungal treated rice husk were determined using the DNS method by Miller (1959). The carboxymethylcellulase activities were carried out by measuring the amount of reducing sugar released from CM-cellulose. A reaction mixture containing 0.2 ml supernatant (crude enzyme) and 1.3ml 2% (w/v) CM-cellulose in 0.1M acetate buffer pH 5.0 was incubated at 30^{o}C, 40^{o}C, 50^{o}C, 60^{o}C for 60 minutes. The reaction was quenched by cooling on ice and the amount of reducing sugar measured by dinitrosalicylic (DNS) acid procedure (Miller, 1965). The mixtures were then incubated at 100^{o}C for 5 minutes. Subsequently, the reactions were stopped by cooling on ice. Distilled water was also added into the final volume to make up a total of 16ml and the colour intensity measured at wavelength of 550 nm. The non enzymatic release of sugar was corrected by setting up a separate blank for each sample. One unit (U or μmol/min) of CM-cellulose

activity is defined as the amount of enzyme that produced one μmol of reducing sugar as glucose per minute under the assay condition.

Determination of Incubation time on Carboxymehtylcellulase activity

Test for carboxymethylcellulase activity was carried out on days 1 (24 hours), 2 (48 hours), 3 (72 hours), 4 (96 hours), 5 (120 hours), 6 (144 hours) and 7 (168 hours) at optimal temperature of 30^{o}C and pH 5.

Determination of optimal incubation temperature for carboxymethylcellulase activity

The effect of incubation temperature on activity of carboxymethylcellulase produced by the fungal isolated monocultures (*Aspergillus fumigatus* (AF) and Aspergillus *niger* (AN)) were studied by determining the released sugar using the DNS Method by Miller (1959) at various temperature ranges of 30^{o}C, 40^{o}C, 50^{o}C, 60^{o}C for 60 minutes.

Determination of optimal incubation pH for Carboxymethylcellulase activity

Carboxymethylcellulase activity by the two fungal isolates was determined at various pH range 4, 5, 6, 7, 8, 9 and 10 using 0.1M acetate buffer with the pH adjustments carried out using HCl (0.1N) and 0.1N NaOH to achieve acidity and alkalinity respectively for 60 Minutes at temperature of 30^{o}C.

Protein Assay

The protein contents of the samples were determined using the method of biuret as applied by Ezeonu (2010), Oyeleke *et al.* (2010) and described by Jayaraman (1981). To 4ml of each fungal treated rice husk filtrate was added 6ml of Biuret's reagent in a test tube. The contents were mixed well and the tubes kept at 37^{o}C for 10 minute during which purple colour developed. The optical density of each tube was measured at 540nm adjustment were carried out using the reagent blank. The concentrations of protein in the enzyme samples were determined with reference to Standard Bovine Serum Albumin.

Fermentation for Ethanol Production Using Baker's Yeast and Yeast from Palmwine

Culture filtrate of the fungal treated rice husk was inoculated with Baker's yeast (*Saccharomyces cerevisiae*) and allowed to ferment for seven days (Sandhu *et al.,* 1998). Spectrophotometric estimation of percentage ethanol produced was determined.

Glucose Yeast Extract medium was prepared by dissolving 10 gm of glucose, 0.1 gm of potassium bisulfate (KH_2PO_4), 0.5 gm of ammonium sulfate $(NH_4)_2SO_4$, 0.1 gm of yeast extract and 0.05 gm of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) in some amount of distilled water and made final volume to 100 ml with distilled water. pH of the medium was maintained at 4.6.

The yeast cells were grown by inoculating 1gm of baker's yeast into the medium prepared above. The nutrient medium was incubated at 37^oC for 48 hours with 120 rpm agitation using a horizontal shaker. After 48 hours, 1ml of yeast suspension was taken from the medium of the grown yeast and centrifuged at 10000 rpm for 10 minutes, the pellets were collected and 5 ml of sterile distilled water added to it, this was centrifuged at 10000 rpm for 10 minutes and the harvested yeast pellets used for the inoculation of a fungal treated rice husk (filtrate) broth. This method was repeated and adopted for each fungal treated rice husk filtrate.

Sample of fresh palm wine was obtained and allowed to ferment for 48 hours, at 37^oC with 120 rpm agitation using a horizontal shaker (similar to that carried out using the baker's yeast inoculated medium). After 48 hours, 1ml of the palm wine suspension was taken and centrifuged at 10000 rpm for 10 minutes. The pellets were collected and 5 ml of sterile distilled water added to it, this was centrifuged at 10000 rpm for 10 minutes and used for inoculation of the fungal hydrolyzed rice husk (filtrate) broth. The above step was carried out for each treatment. The fermentation was carried out using the prepared yeast as explained above. To 20 ml of the saccharified fungal treated rice husk filtrate (SAFUTRIHUFI) in 250 ml conical flask was added 2ml of the baker's yeast culture and another set of fermentation setup was administered using 2ml of yeast from the palm wine. The treatment of the fermentation experiment for both the baker's yeast and palm wine yeast was carried out in all the samples in duplicate. The flask were tightly plugged with cotton wool and sealed with air tight elastic plastic materials (balloons) and left to ferment for 7 days at 30^oC with 90 minutes agitation at 120 rpm daily.

To determine the quantity of alcohol produced, distillation was carried out by the following methods: Potassium dichromate ($K_2Cr_2O_7$) solution was prepared by dissolving 33.76 gm of $K_2Cr_2O_7$ in 400ml distilled water and 325 ml concentrated sulphuric acid, cooled and then made total volume to 1 liter with distilled water. This solution was kept refrigerated for 10 minutes before use. To measured 20 ml of saccharified alcohol fermented filtrate (SAFF) of 7 days incubation period of each of the fermented fungal treated filtrate was added 29 ml of distilled water and distilled at 70^oC. Each sample (SAFF) was then distilled for 2 hours (using a distillation apparatus) and the distillate collected in a 100 ml beaker containing 25ml of $K_2Cr_2O_7$

solution. The distillate (25 ml) of each sample was then incubated at 60^{o}C for 20 minutes in a water bath. From the distillate solutions, 5ml of each sample (distillate) was diluted with 5ml of distilled water (1:1 dilution).

The optical density at 600 nm was measured using a spectrophotometer. The percentage alcohol was determined using a standard curve. In preparing the standard curve, different dilutions of ethanol solution were prepared by mixing stock ethanol with distilled water to achieve 5 % solution and diluted to 10 ml with distilled water in the test tube. The range of ethanol percentage was 0.2 to 1 %. The various percentages of ethanol for the standard curve were made up to 2 ml by addition of $K_2Cr_2O_7$ and thoroughly mixed followed by boiling vigorously for 10 minutes in a boiling water bath. The solutions were cooled for 10 minutes and absorbance read at 600nm in a spectrophotometer. Results obtained from the study were subjected to analysis of variance.

Analysis of Treated Rice Husk

Estimation of reducing sugar

This was determined by the Dinitrosalicylic acid (DNS) method as described by Miller (1959) using glucose in establishing the standard curve. The DNS method is simple, sensitive and adoptable during handling of a large number of samples at a time. In preparation of Dinitrosalicylic reagent (DNS reagent), a solution was formed consisting of 3,5-Dinitrosalicylic acid (1g), 200mg crystalline phenol and 500mg sodium sulphite dissolved by stirring in 100ml 1% NaOH. The reagent was stored at 4^{o}C for at least 2 hours before use. Since the reagent deteriorates due to presence of sodium sulphite, sodium sulphite was added at the time of use. A solution of Rochelle salt (potassium sodium tartarate) was also prepared. From each of the rice husks (both Aspergillus treated and untreated rice husk- fig. 2) 100mg was weighed and sugar extracted from each using hot 80 % ethanol twice (5 ml each time). The supernatant (top/decanted liquid) was collected after the sugar extraction and evaporated by being kept on a water bath at 80^{o}C. Distilled water (10 ml) was added to each test tube to dissolve the sugar by agitation. From the extracted sugar, 3 ml was pipetted out into test tubes and 3 ml of distilled water added to each test tube containing the extracted sugar. To each of these solutions were added 3ml of DNS reagent and heated in a boiling water bath for 5 minutes. While the contents of the tubes were still warm after boiling for 5 minutes, 1ml of the prepared 40% Rochelle salt solution was added to each treatment. The reaction was cooled and the intensity of dark red colour read at 510 nm spectrophotometrically. In readiness for the standard curve, standard glucose solution stock was prepared by dissolving 100mg glucose in 100ml distilled water. For the working standard, 10ml of stock solution was diluted to 100 ml with distilled

water. A series of standards were determined using glucose (0.2 – 1 ml) and a graph plotted. The amount of reducing sugar present in each treatment was extrapolated and calculated.

Calculation

Absorbance corresponding to 0.1ml of test = x mg of glucose

100 ml contains = $\left(\frac{\chi}{0.1} X 100mg \right)$ of glucose = % of reducing sugars.

Estimation of Total Sugar (Carbohydrate)

The carbohydrate content was determined by the phenol sulphuric acid method as described by Dubois *et al*. (1956). In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This forms a green coloured product with phenol and has absorption maximum at 490 nm. Phenol (5%) was produced from redistilled (reagent grade). Thus, 5 g of phenol salt was dissolved in distilled water and diluted to 100 ml. Sulphuric acid 96 % reagent grade was also used in the protocool. Weighed 100mg of the rice husk samples (treatments and controls) were place into different test tubes and hydrolysis was carried out on each sample by keeping samples in a boiling water bath for three hours after addition of 5 ml of 96 % sulphuric acid and thereafter, cooled to room temperature. Neutralization of each solution which involved addition of solid sodium carbonate until the effervescence ceases was carried out on the cooling samples. The volume of each sample solution was made up to 100 ml and 4 ml of each centrifuged. Measured volume of 0.2 ml of the sample solution (supernatant) was pipetted into a test tube and the volume in each tube was made up to 1ml with water. Phenol solution (5 ml) was added to each test tube. After 10 minutes, contents in the tubes were agitated and placed in a water bath at 30^{o}C for 20 minutes for colour development. The contents were read at 490 nm. The standard graph was plotted by preparing working standard of glucose by making a stock solution in which 100 mg of glucose was mixed in 100 ml of distilled water and from this stock solution, 10 ml was pipetted into a conical flask and made up to 100 ml with distilled water. Various volumes: 0.2, 0.4, 0.6, 0.8 and 1 ml was pipetted out of the working standard into series of test tubes. Each was made up to 1 ml with distilled water before adding 5 ml of phenol. These solutions in test tubes were placed in water bath at 30^{o}C for 20 minutes. A blank of 1ml distilled water was also prepared to which 5ml phenol was added. The colour developed was read at 490 nm using a spectrophotometer. A standard graph was then plotted. The amount of total carbohydrate present in the sample solution was calculated using the standard graph.

Calculation

Absorbance corresponds to 0.1 ml of the test = *x* mg of glucose.

100 ml of the sample solution contains = $\left(\frac{\chi}{0.1} X 100mg\right)$ of glucose

= % of total carbohydrate present.

Non Reducing Sugar: This was obtained by subtracting the values of the reducing sugar from the total sugar.

Measurement of Cellulose Content of Fungal and Non-Fungal Treated Rice Husk

Cellulose content of fungal and non-fungal treated rice husk was determined according to the modified gravimetric method of Marzieh and Marjan (2010). Each group of heat and fungal treated rice husk biomass and two controls weighing 0.5 g was transferred to 150 ml flat bottom flask component of a soxlet extractor apparatus and 25 ml of 96 % ethyl alcohol and 13 ml of 65 % nitric acid were added. The experiments were carried out in triplicate. The apparatus was set up to allow for condensing of volatile solvents and heated for 1 hour. After hydrolysis, flask contents were filtered. Once more, remaining cellulose on the filter paper was transferred into the flask. This step was repeated similarly to the previous stage. This process was repeated for the third time making the total period of one complete measurement 3 hours. The cellulose with the filter paper (figure 2b) was dried to constant weight at 102^{o}C. The cellulose content was calculated from the following equation (Oakley, 1984; Ritter and Fleck, 1924).

$$\text{Cellulose (\%)} = \frac{Cellulose\ dry\ weight}{Sample\ dry\ weight} \times 100$$

Figure 2: Fungal treated rice husk and the produced Cellulose.

Determination of lignin contents of heat treated and fungal hydrolyzed rice husk (modified Klason Lignin Determination method).

Samples from each group (heat treated and fungal *(Aspergillus fumigatus, Aspergillus niger)* treated rice husk, non-fungal but heat treated rice husk -control C1 and non-heat and non-fungal treated rice husk- C2) of rice husks weighing 150 mg (0.15 g) in triplicate were impregnated with 3

ml of 72 % Sulphuric acid and placed in a bath with a controlled temperature of 30^{o}C for one hour. The step above was followed with the addition of 68 ml of deionised water and the samples were placed in autoclave at 121^{o}C for 1 hour 15 minutes. Thereafter, the samples were cooled and the lignin filtered using a filter paper. Insoluble lignin was washed with deionised water until neutral pH and then dried in an oven at 103^{o}C until constant weight.

The lignin content was calculated by the following formula:

$$\text{Insoluble Lignin (IL) \%} = \frac{\text{W lignin}}{\text{W fiber}} \times 100$$

Where IL = Insoluble lignin content (%);

W lignin = Oven dry weight of the insoluble lignin or Klason lignin (g)

W fiber = Oven dry weight of rice husks

Spectrophotometric Method for Determination of Soluble Lignin

The filtrate obtained by the modified Klason lignin was used to determine the soluble lignin content in sulphuric acid by the spectrophotometric method. In this method, 5ml of 3 % Sulphuric acid was added to 5 ml of the filtrate. A spectrophotometer UV was used to measure the absorbance of the solution at a wavelength of 330 nm. Therefore, the soluble lignin content was calculated by the following formula:

$$\text{SL (\%)} = \frac{CV}{1000 \times W\ fiber}\ X\ 100$$

Where SL = Soluble lignin content (%);

C = Concentration of soluble lignin in the filtrate (g/l)

V = Total volume of the filtrate (ml)

W fiber = Oven dry weight of rice husk (g).

The concentration (C) of soluble lignin in the filtrate is given by:

$$C = \left(\frac{A}{177}\right)\left(\frac{Vfinal}{Vinitial}\right)$$

Where A = Absorbance at a wavelength of 330nm;

V final = Final volume of the solution (ml)

V initial = Initial volume of the solution (ml).

The total lignin content was obtained by the addition of insoluble and soluble lignin obtained by both methods.

Thus, Total Lignin (TL) % = Insoluble lignin (IL) (%) + Soluble lignin (SL) %.

Estimation of Hemicellulose by Neutral Detergent Fibre (NDF)

The estimation of hemicellulose was done according to the method of Goering and Vansoest (1975).

The neutral detergent solution was prepared by weighing 18.6 g disodium ethlenediamine tetraacetate and 6.81 g sodium borate decahydrate into a beaker and dissolving in 200 ml of distilled water by heating. A 150 ml solution containing 30 g of sodium lauryl sulphate and 10 ml of 2-ethoxy ethanol was also added and thoroughly mixed. To the whole solution prepared above was added 100 ml of 4.5 g of disodium hydrogen phosphate. The total volume was made up to one litre and the pH adjusted to 7.0. To 1 g of each powdered sample (dried fungal treated rice husk and controls) in a refluxing flask was added 10 ml of cold neutral detergent solution, 2 ml of decahydrinaphtalene and 0.5 g of sodium sulphite. The solution was heated to boiling and refluxed for 60 minutes. The contents were filtered through Watman 1 filter paper while hot and the residue component washed with hot water. Additional two more washing was carried out using acetone and the final residue transferred to a crucible cooled in a desiccator and dried at 100^{o}C for 8 hours. Refluxing the sample material with neutral detergent solution removes the water-solubles and materials other than the fibrous component. The left out material is weighed after filtration and expressed as Neutral Detergent Fibre (NDF).

Calculation

Hemicellulose = Neutral detergent fibre (NDF) - Acid detergent fibre (ADF)

NB: Acid detergent fibre value is the same as Lignin value.

Results and discussion

The effect of duration on carboxymethylcellulase (CMC) activity of the crude enzyme of the various treated rice husk were investigated at 30^{o}C for 60 minutes at pH 5. The result of figure 3 showed that the crude enzyme of *Aspergillus fumigatus* treated rice husk showed increased activities of 53.49 ± 3.74 µg/ml/min, 55.34 ± 2.92 µg/ml/min, 57.62 ± 2.49 µg/ml/min and 65.26 ± 7.12 µg/ml/min at 72 hours, 120 hours, 144 hours and 168 hours. However, the aforementioned activities each with high CMC activity have no significant increase at $p > 0.05$ to each other and thus any of these durations is suitable for hydrolysis of rice husk with *Aspergillus fumigatus* even though 168 hours showed higher activity than the rest. This result agrees with similar work by Bharathi and Ravindra (2006) in which solid state fermentation of rice husk was carried out using isolated fungal strain *Scopulariopsis* MTCC 3553 (different strain from *Aspergillus fumigatus*) and cellulase activity was expressed in terms of percentage solubilisation of whatman paper and in the sixth day (144 hours) of the ten days experiment maximum activity of crude cellulase enzyme extract of 16.7 % was recorded. Each of these activity values gave

significant increase to the CMC activity of 25.24 ± 3.68 µg/ml/min gotten after 24 hour at $p < 0.05$ as the least activity. *Aspergillus fumigatus* showed a remarkable decrease in carboxymethylcellulase activity after 96 hours (Figure 3) with a value of 28.22 ± 3.74 µg/ml/min slightly above the non heat and non fungal treated rice husk with a value of 22.93 ± 1.66 µg/ml/min. The result gotten here is also in conformity with the work of Svarachorn (1999), which showed that optimal condition for both Xylanase activity of 16.8 x 10^4 unit/g rice straw and cellulose activity of 3.4 x 10^3 unit/g rice husk was obtained when treated with *Aspergillus fumigatus* after 6 days (144 hours) of incubation. Also crude enzyme of *Aspergillus niger* treated rice husk gave higher carboxymethylcellulase activities of 44.69 ± 7.23 µg/ml/min, 40.56 ± 9.13 µg/ml/min and 43.50 ± 3.74 µg/ml/min at 96 hours, 120 hours and 168 hours and therefore support hydrolysis of rice husk at any of these incubation duration even thought the duration of choice from the finding is 96 hours. The result of this work is not in conformity with that of Okafoagu and Nzelibe (2006) in which rice husks were first pre-treated with sulphuric acid before fungal pre-treatment with *Aspergillus niger*. The CM-cellulase value (88.2 ± 2.3 IU) of their result after 96 hour was twice that of this experiment (44.96 ± 0.83 µg/ml/min). This is definitely due to hydrolysis with sulphuric acid. The non fungal and non heat treated rice husk was used as control and gave the highest CMC activity value of 22.93 ± 1.66 µg/ml/min at 96 hours which showed significant decrease from the CMC activities of the crude enzyme of *Aspergillus niger* treated rice husk as illustrated in figure 3. The real CMC values of crude enzymes of *Aspergillus fumigatus* and *Aspergillus niger* treated rice husk from this work, could be obtained by subtracting the corresponding values according to duration from the non fungal, non heat treated rice husk CMC activities. *Aspergillus fumigatus* is the fungus of choice and could be used in rice husk treatment for extented period of time such as from 48 hours to above 168 hours.

Figure 4, illustrates the effect of temperature on carboxymethylcellulase activities. At pH of 5 and for 60 minutes duration the highest carboxymethylcellulase activities of 61.14 ± 5.38 µg/ml/min and 59.96 ± 8.45 µg/ml/min from crude enzymes of *Aspergillus fumigatus* and *Aspergillus niger* treated rice husk was obtained at 30^{o}C. These values showed non significant increase to CMC activities of 58.79 ± 5.82 µg/ml/min and 58.79 ± 2.57 µg/ml/min at 50^{o}C and 80^{o}C from crude enzyme of *Aspergillus niger* treated rice husk. This finding notwithstanding still makes 30^{o}C the best temperature for CMC activities of the crude enzyme of *Aspergillus spp* used in this experiment as illustrated in figure.4. Ghadi *et al.* (2011) presented results which are in agreement to those of this work and clearly illustrated that the maximum activity of the fungus used in rice husk solid fermentation pre-treatment at which the optimum liquid glucose was

produced was recorded at 30^{o}C. Also Hallesmeersch and Vandamme (2003) in a research to degrade grass cell wall by fungal cellulases and hemicellulases discovered that the optimal growth temperature of the ascomycetous fungus (unidentified) P13, was found to be 30^{o}C and less growth was noticed at 25^{o}C, while no growth occurred at 37^{o}C by this fungus. Milala *et al*. (2005); Okafoagu and Nzelibe (2006) using *Aspergillus niger* on various agricultural wastes (Garcinia kola, Guinea Corn leaves, Maize cobs, Millet and Rice husk) to determine optimal conditions for cellulase, CM-cellulase and β-glucosidase production based their estimations on temperature value of 30^{o}C. All the results of the research are in concordance with this research result with optimal temperature of 30^{o}C for optimum carboxymethylcellulase activities of *Aspergillus fumigatus* and *Aspergillus niger* respectively.

At 30^{o}C for 60 minutes activity duration, crude enzymes of *Aspergillus fumigatus* treated rice husk gave higher carboxymethylcellulase activities of 63.49 ± 7.30 µg/ml/min, 61.14 ± 8.90 µg/ml/min and 59.96 ± 9.80 µg/ml/min at pH of 5, 6 and 7. These values showed no significant increase in CMC activity at $p < 0.05$ from each other even though the highest activity was at pH 5. Using rice as substrate and only *Aspergillus niger* for treatment, Milala *et al* (2005) had a different result from that obtained in this work in which maximum activity was recorded at pH 4. However, Quiroz-Castañeda *et al*. (2008) in their work demonstrated that the highest cellulytic activity from *B. adusta* in wheat straw medium was detected at pH of 5 with no significant differences observed at pH 4 and 6. Similarly, they observed that *P. sanfuineus* preferred pH 5 for maximum CMC activity levels as higher values of pH reduced the activity dramatically to less than one third, while at pH 6 the activity was one half of that shown at pH 5. Moreso, crude enzymes of *Aspergillus niger* treated rice husk gave the highest carboxymethylcellulase activities of 68.20 ± 8.63 µg/ml/min and 65.84 ± 5.27 µg/ml/min at pH 5 and 9 respectively as illustrated in figure 5. The curve (figure 5) showed that pH 5 is the best pH from the result of this work. Hallesmeersch and Vandamme (2003) in their work reported that an ascomycetous fungus P13 thrived at pH of 7 up to pH 9 which support some of the findings in this experiment as seen in figure 5 where *Aspergillus fumigatus* and *Aspergillus niger* showed high carboxymethylcellulase activities at both pH which comes next to pH 5 in each group.

Release of cellulose from rice husk by heat treatment alone was not effective. Cellulose determination from heated but non fungal treated rice husk (C1) gave 27 ± 5.44 % and non fungal and non heat treated rice husk (C2) yielded 22 ± 1.63 % showing a 4 % cellulose difference which is statistically not significant at $P > 0.05$. This is in agreement to observation made by Bharathi and Ravindra (2006) that biodegradation of native

untreated cellulose is very slow, thus heat treatment help to convert cellulose from its crystalline form to its amorphous component for ease of hydrolysis. Also in agreement to this result was a statement by Wyman *et al*. (2005) that physical-chemical pretreatments (such as steam explosion, with or without diluted sulfuric acid) are used to loosen lignin and fibrils of cellulose and monomers of hemicellulose components. However, heat treatment in combination with the fungal treatment ensured that more delignification took place making the cellulose to be abundantly available as the cell wall released the cellulose and hemicellulose components available in the rice husk. Thus, cellulose yield by *Aspergillus fumigatus* treated rice husk is 45 ± 3.31 %, *Aspergillus niger* treated rice husk gave 40 ± 9.43 % cellulose. All these values showed significant increase at $p < 0.05$ when compared to the two controls (C1 and C2). However, di-culture combination of *Aspergillus fumigatus* and *Aspergillus niger* (AF+AN) yielded 33 ± 5.44 % which did now give any significant increase in cellulose yield at $p > 0.05$ when compared to heated but non fungal treated rice husk (C1) with 27 ± 5.44 % cellulose yield. The listed monoculture treatments gave better yield of cellulose and are in agreement to work carried out by Belewu and Babalola (2009) in which rice husk was treated with *Rhizopus oligosporus* and they achieved 38.62 % cellulose from treatment as against untreated rice husk value of 33.39 %. Hence fungal treatment is efficient in the production of cellulose from rice husk especially when in combination with heat treatment of rice husk. These two methods caused separation of lignocellulosic components by breaking the hydrogen bonds between cellulose and the hemicellulose-lignin ester cross links in the rice husk. Concerted hydrolytic effort of both heat and fungal treatments having various endo and exo-lignocellulosic enzymes has great impact on breaking the protective lignin sheath as well as the crystalline structure characteristic of the lignocellulose to release the cellulose as seen in the result of this experiment. For the insoluble lignin determination carried out in this research, a general statistical descriptive pattern was established in which *Aspergillus niger* and diculture combination of *Aspergillus fumigatus* and *Aspergillus niger* with insoluble lignin yield of 26.67 ± 6.26 % and 33.33 ± 8.32 % respectively from treated rice husk gave mean values that were statistically significant at $P < 0.05$ when compared to both controls (C1 = non fungal but heated rice husk (13.33 ± 3.14 %) and C2 = totally untreated rice husks (11.11 ± 3.63 %) respectively. However, *Aspergillus fumigatus* treated rice husk with an insoluble lignin yield of 15.55 ± 1.82 % did not give any significant increase in insoluble lignin at $p > 0.05$. The highest soluble lignin value of 0.012 ± 0.00 % was obtained from *Aspergillus fumigatus* (AF) treated rice husk. All the values obtained in the soluble lignin in this experiment are quite

negligible as seen in figure 6. Therefore, the deductions made for the insoluble lignin applies to the total lignin.

The result of this work showed that all the fungal treated rice husk had percentage values of carbohydrate which has no significant increase at P > 0.05 when compared with the two controls; heated but non fungal treated rice husk (C1) and non heated and non fungal treated rice husk (C2). In figure 5, the percentage mean values of carbohydrate yield of some fungal treatments in this category include: *Aspergillus niger* (AN) = 12.49 ± 2.75 %, *Aspergillus fumigatus* and di-culture of *Aspergillus fumigatus* and *Aspergillus niger* (AF+AN) has 19.52 ± 10.05 % and 14.78 ± 3.30 % respectively. Heating alone was not enough to release the reducing sugar in appreciable quantities from the rice husk. Worthy of note is the established fact from this finding that the entire fungal treated rice husks (*Aspergillus fumigatus* treated rice husk = 2.60 ± 0.30 %; *Aspergillus niger* treated rice husk = 2.32 ± 0.13 % and diculture AF+AN treated rice husk = 2.54 ± 0.20 %) gave percentage reducing sugar values which showed statistical significant increase at P < 0.05 level of significance when in comparison to the two controls C1 and C2 with reducing sugar yield of 1.61 ± 0.21 % and 1.21 ± 0.05 % respectively. This is in agreement with the work of Patel *et al*. (2007) in which rice husk treated with two fungi: *Aspergillus awamori* and *Pleurotus sajor-caju* gave good reducing sugar yield of 14.3 mg/g and 15.35 mg/g which was significantly different at P < 0.05 level of significance in comparison to the control (untreated rice husk) which had a value of 2.6 mg/g. These values even thought statistically significant, are quite small compared to the bulk of rice husk from which they were hydrolyzed. Figure 6 gave the values of the highest mean percentage contents of non reducing sugar from the treatments in the following order: *Aspergillus fumigatus* (AF) = 16.52 ± 3.53 %, combination of *Aspergillus fumigatus* (AF) and *Aspergillus niger* (AN) = 12.25 ± 3.10 %. The least value of the non reducing sugar from the experiment is 10.17 ± 2.62 % from *Aspergillus niger* (AN) treated rice husk. All the values showed significant increase in non-reducing sugar yield at p < 0.05 except the value of the *Aspergillus niger* treated rice husk. This result is not in concordance with the work carried out by Patel *et al*. (2007) in which the values of the reducing sugar was higher than those of the non reducing sugar. In the experiment most fungal treated rice husk especially those listed above with high mean percentage contents of non reducing sugar values showed statistical significant increase at P < 0.05 level of significance. The higher contents of non reducing sugar compared to the reducing sugar was expected as explained by Okaforagu and Nzelibe (2006) that for reducing sugar to be formed concerted efforts of three enzymes with specific functions must come into play. For instance, endo-β-glucanase (1,4-β-D-glucan glucanohydrolase) acts randomly on

cellulose chains yielding glucose (reducing sugar) and cello-oligosaccharides. Due to this random action less reducing sugar may likely result as seen in the results of this experiment. Also exo-β-glucanase (1,4-β-D-glucan cellobiohydrolase or Avicellase) attacks the non-reducing end of cellulose yielding higher quantities of cellobiose (non-reducing sugar). Thus for more reducing sugar to result which was the limitation of the enzymes acting from the fungal treated rice in this experiment, enzymes such as β-glucosidase (cellobiase) must be robust and plentiful in the fungal treated rice husk broth so as to fully hydrolyze cellobiose to glucose. Heat treatment no doubt helped in the hydrolysis of non-reducing sugar to reducing sugar in the experiment, but the rate was likely minimal to gain appreciable quantity of reducing sugar. The percentage yield of hemicellulose from the various fungal treated rice husks worthy of note and which showed significant increase at $P > 0.05$ level of significance especially when compared to 19.00 ± 5.00 % (C1 heat treated sample only) was from *Aspergillus fumigatus* (AF) treated rice husk yielding 29.00 ± 6.00 %. Other fungal treated rice husk whose percentage yields showed non-significant increase at $P > 0.05$ in comparison to the yield of heat treated rice husk-C1 but gave significantly increase when compared to non heated and non fungal treated rice husk- C2) with a percentage hemicellulose yield of 17.00 ± 3.00 %, include: 28.00 ± 0.00 % from *Aspergillus fumigatus* and *Aspergillus niger* (AF + AN) treated rice husk and 28.50 ± 1.50 % hemicellulose yield from *Aspergillus niger* treated rice husk. Belewu and Babalola (2009) recorded 19.05 % hemicellulose from rice husk treated with *Rhizopus oligosporum* as against the initial hemicellulose content of 14.67 % of the untreated rice husk. Their work is quite a departure from the values gotten from this work since all the percentage hemicellulose contents recorded in figure 6 had reasonable higher values. Generally, *Aspergillus spp* as shown in figure 6 cannot be associated with release of protein from the rice husk as shown in the illustrations of the experimental results. This is not in agreement with work carried out by Ghadi *et al*. (2011) in which they explained that crude protein always increases in fermented rice husk. Heat has little effect on availability of protein as can be seen from the results of the controls; as a matter of fact it can denature the available protein and cause its non availability.

Ethanol yield from rice husk treated with *Aspergillus fumigatus* and fermented with yeast from palmwine gave a yield of 6.60 ± 0.48 % and that from *Aspergillus niger* treated rice husk fermented with same yeast (from palm wine) gave a yield of 6.46 ± 0.39 %. These values showed significant increase at $P < 0.05$ compared to those of the controls and that of the diculture yield as shown in figure 6. The fungal treated rice husk (*Aspergillus fumigatus* (AF), *Aspergillus niger* (AN)) fermented with bakers yeast gave values of 5.60 ± 0.42 % and 4.37 ± 0.02 % while that of diculture

(*Aspergillus fumigatus* and *Aspergillus niger* (AF+AN) yielded 4.86 ± 0.16 % of ethanol. These values gave significant ethanol increase compared to those of controls (Heated but non fungal treated rice husk (C1) and non heated and non fungal treated rice husk (C2)) with values of 3.05 ± 0.03 % and 2.24 ± 0.12 % respectively. The values of ethanol gotten from the result of this research are quite different than those obtained by Patel *et al*. (2007). They obtained 1 g/litre of ethanol from *Aspergillus niger* (AN) treated rice husk using baker's yeast. This is quite low compared to the bio-ethanol obtained from this experiment. Thus, *Saccharomyces cerevisae* (yeast) from palm wine is ideal in production of bio-ethanol from rice husk with any of the *Aspergillus* fungi as the choice in hydrolysis of rice husk (figure 6).

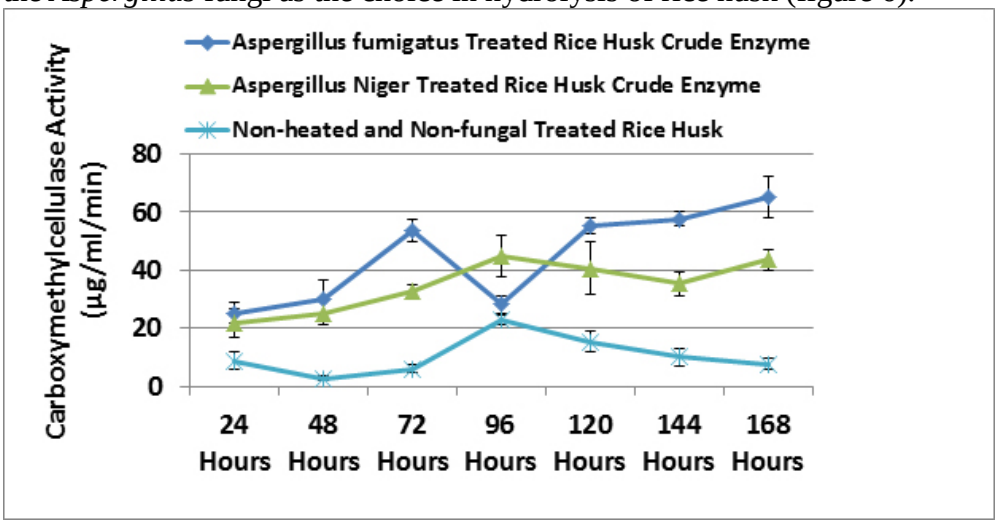

Figure 3 : Effect of incubation duration on carboxymethylcellulase activity of *Aspergillus spp* crude enzyme treated and non fungal treated rice husk.

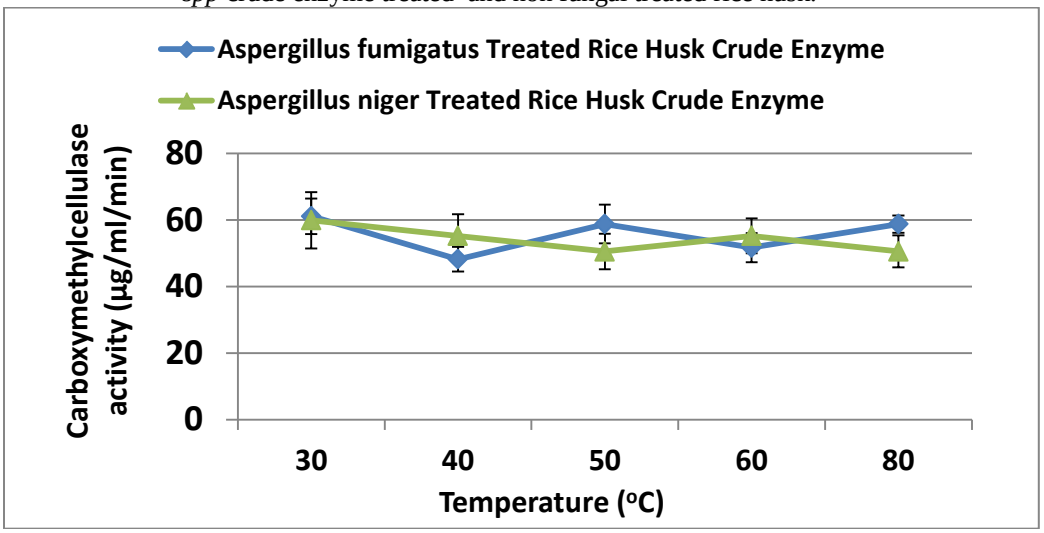

Fig. 4: Effect of temperature on Carboxymethylcellulase activity of crude enzyme *of Aspergillus spp* treated and non fungal treated rice husk.

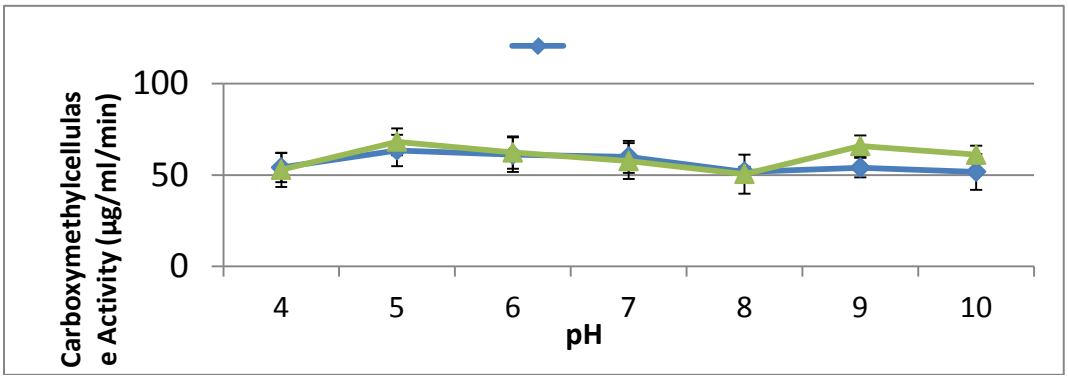

Fig. 5: Effect of pH on carboxymethylcellulase activity of crude enzymes *of Aspergillus spp* treated and non fungal treated rice husk.

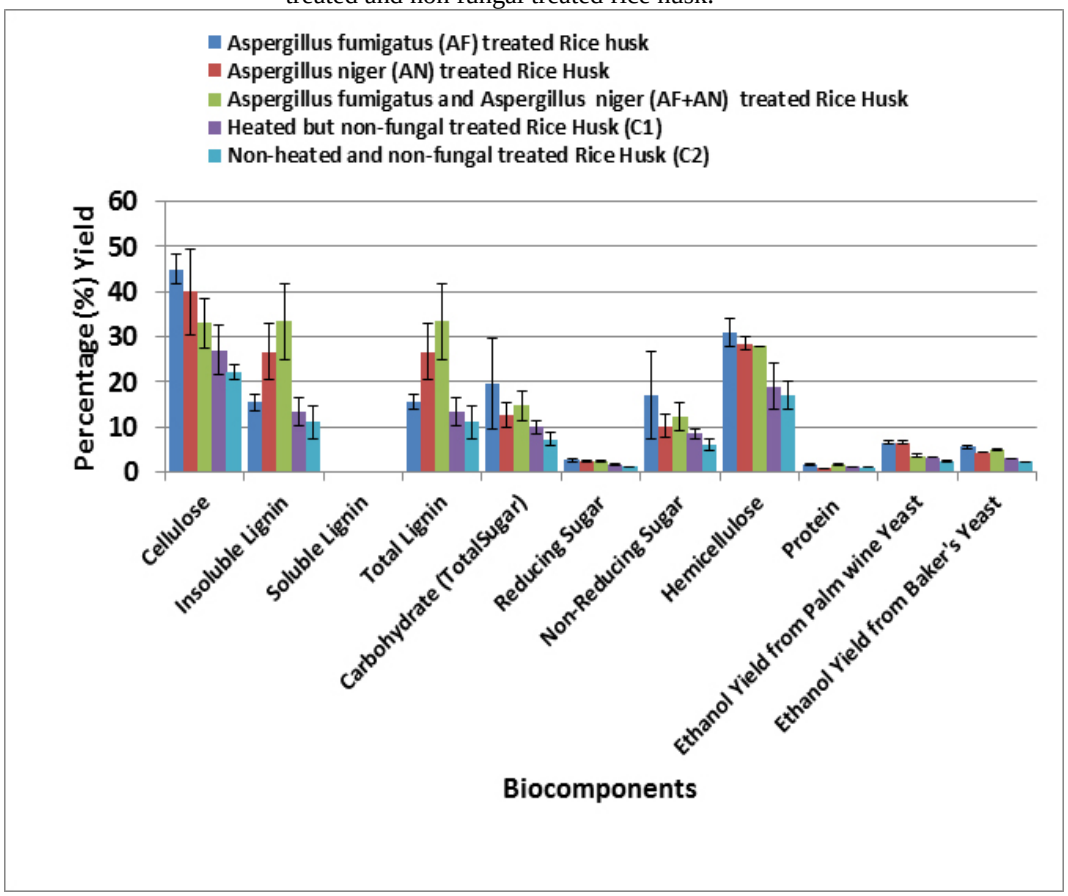

Fig. 6 : Effect of *Aspergillus fumigatus* , *Aspergillus niger* and heat treatment on enhancement of bio-components of rice husk.

Conclusion

The values obtained in the various bio-fuel and biomass components of fungal monocultures of *Aspergillus fumigatus* (AF) and *Aspergillus niger*

(AN) treated rice husk had no significant difference in increase of product yield at $P > 0.05$. However, there were significant differences in product yield between the fungal treated rice husks and those of the controls at $P < 0.05$. The optimized condition involved in bio-product estimation showed that 30^{o}C and pH 5 gave the maximum carboxymethylcellulase activity in crude enzyme of products respectively. It was discovered that heat pre-treated rice husks with additional hydrolysis using the fungi (*Aspergillus fumigatus* and *Aspergillus niger*) showed increase in biomass and biofuel yields. Therefore bioethanol and other biomass components is obtainable from rice husk *ipso facto* other agro-grain processed wastes when treated with heat, enzyme hydrolysis (with the carefully selected fungi consortia such as the one understudied) and properly channeled fermentation procedures.

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